

Nonselective Biochemical Methods for the
Determination of Fungal Mass and Community
Structure in Estuarine Detrital Microflora

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Nonselective Biochemical Methods for the Determination of Fungal Mass and Community Structure in Estuarine Detrital Microflora

D. C. White, R. J. Bobbie, J. S. Nickels, S. D. Fazio and W. M. Davis

Department of Biological Science, Florida State University, Tallahassee, Florida 32306, USA

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Abstract

The determination of biomass and community structure of the fungal community in estuarine detritus by cultural or extractive methods can be incomplete and selective. Biochemical measures could provide a less biased estimate of the fungal components if they can be proved reliable. Assay of lipid and cell wall components can readily provide differentiation in reciprocal mixtures of bacteria and fungal monocultures. To test natural microbial assemblies, small plastic sheets were exposed in a subtropical estuary. The sheets were then transferred to a laboratory where estuarine environments that inhibited prokaryotic growth and stimulated fungal growth were compared with an environment where eukaryotic growth was inhibited and prokaryotic growth was stimulated. The morphology determined by scanning electron microscopy showed typical mycelial networks where eukaryotic growth was stimulated and none where inhibited. The stimulation of eukaryotic growth produced a greater biomass measured in terms of lipid phosphate, respiratory activity or extractable adenosine nucleotides, but with a slower synthesis of phospholipids and DNA and a smaller concentration of muramic acid (a unique prokaryote wall component). The stimulation of eukaryotic growth increased the rate of sulpholipid synthesis relative to DNA or phospholipid synthesis.

The prokaryote-stimulated environment yielded much higher proportions of iso and anteiso pentadecanoic, cis vaccenic, 17 and 19 carbon cyclopropane fatty acids in the lipids. The eukaryotic-stimulated environment showed increased 18 carbon dienoic, polyenoic of both the γ and α linolenic series and the long (greater than 22 carbon) fatty acids. The prokaryote-stimulated environment showed significantly higher levels of lipid ribose, arabinose, xylose, glucose and several unknown neutral carbohydrates compared with much elevated lipid inositol in the eukaryote-stimulated environment. The prokaryote-stimulated environment yielded a lipid extract rich in ethanolamine and lysine; the eukaryote-stimulated environment yielded lipid enriched in serine.

The steroids as expected were predominant in the eukaryote-stimulated microflora. Two estuarine fungi grown in monoculture showed similar lipid profiles to the eukaryote-stimulated microcosm, suggesting the biochemical measures initiated in this study can lead to a deeper understanding of the role of fungi in marine microbial ecosystems.

Introduction

The problems associated with the accurate estimation of the biomass and community structure of bacteria in sediments and detrital communities (reviewed in King and White 1978) are compounded in the analyses of fungi because of the anastomosing filamentous thalli which so effectively penetrate detrital substrates.

Classical plate counts of bacteria in the water column have been known to be considerably less than direct counts since the studies of Butkevich in 1932. The problem of cultural enumeration with fungi is further complicated by the contamination of detrital particles with inactive trapped propagules, which readily overgrow the culture media when plated on nutrient media

(Park 1972a, 1972b, 1974). Surface washing is inadequate to remove these propagules (Bisset and Widden 1972). Surface sterilization may yield more accurate estimates of "active decomposers" (Newell 1976).

There is an even more basic problem, and that is defining what the individual unit should be in a mycelial syncytium (Ainsworth 1968), when fragments can each give rise to colonies.

The standard methods for determining fungal biomass are adaptations of the agar film method of Jones and Mollison (1948) that involves maceration and homogenization, extraction and very careful preparation of agar film and calculation of the hyphal length per unit of substrate. The improvements of Thomas *et al.* (1965),

Frankland (1974, 1975), Hansen *et al.* (1974) and Anderson and Slinger (1975) have helped, but the method is time-consuming and assumptions as to mass per unit of mycelial length are required. Various colorimetric methods of analysis for glucosamine, a component of the fungal wall, can give fungal biomass estimations (LeJohn 1971, Ride and Drysdale 1972, Swift 1973a, Hepper 1977). However, chitin assays are successful only with some higher plant detritus uncontaminated with bacteria or microinvertebrates, whose walls also contain glucosamine polymers (Swift 1973a). Swift (1973b) found that agar film biomass estimations were consistently lower than analyses based on glucosamine from the chitin wall. A closer correlation was achieved by comparing hyphae separated from soil and collected on membrane filters after fluorescent staining to the extractable ATP (Paul and Johnson 1977), but this method still requires separation of the fungal mycelia from their substrates.

With the success of lipid analysis in the examination of the community structure of estuarine sediments (White *et al.* 1979b), a program to help define the fungal portion of the marine detrital microbiota was initiated. The admonition of Swift (1973b) to apply multiple analyses was followed, and instead of relying on relatively nonspecific colorimetric tests the individual components or their derivatives were purified before being measured insofar as possible.

By manipulating a detrital microbial population it was possible to begin to define some of the key components necessary to resolve the fungal population structure as a part of the total microbial assembly.

Materials and Methods

Materials

Nanograde solvents (Burdick and Jackson, Muskegon, Michigan) or freshly redistilled chloroform were used for derivatizations. For extractions analytical grade solvents were used without further purification. Standards were purchased from Supelco, Inc. (Bellefonte, Pa.), Applied Science (State College, Pa.), Aldrich, Inc. (Milwaukee, Wis); Sigma Chemical Co. (St. Louis, Mo.) and Pierce Chemical Co. (Rockford, Ill.).

Scanning electron microscopy

Samples were initially fixed in glutaraldehyde, relaxed in artificial sea water, critical point dried and examined with a Cambridge Stereoscan S4-10 microscope (Cambridge Instrument Co., Ossining, N.Y.) as described by Morrison *et al.* 1977).

Lipid extraction and analysis

The analytical scheme used for this study is diagrammed in Figure 1.

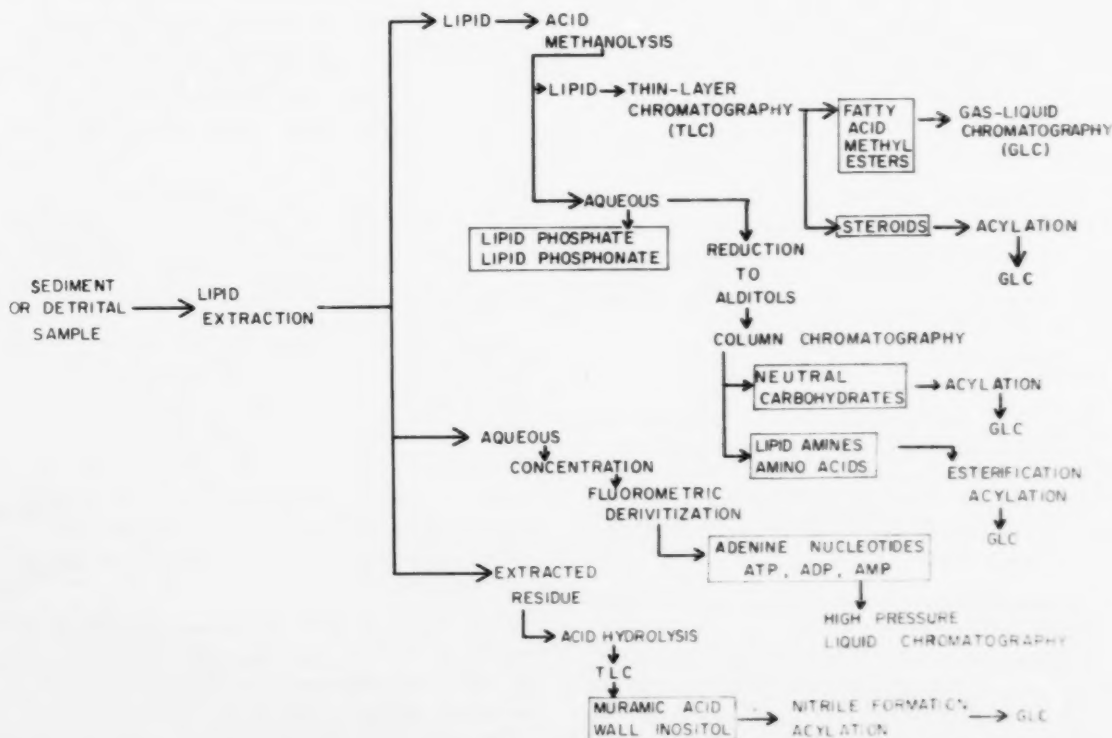


Fig. 1. Diagram of the analytical sequence used to determine the microbial population structure. Components are shown in boxes.

The lipid extraction involves the highly efficient one-phase chloroform-methanol procedure of Bligh and Dyer (1959), as modified for marine detritus and sediments (White *et al.* 1979c). This lipid phase was recovered after filtration through Whatman fluted filter paper, which both removed particulates and dried the lipid. Internal standards were added, the lipids were dried in a stream of nitrogen, redissolved in anhydrous methanol, concentrated HCl, chloroform (9:1:1, V/V) in a screw cap test tube and heated at 100 °C for 60 min. The methanolysate was then partitioned and the lipid phase was applied to thin layer plates (Whatman K6 silica gel) (TLC) and separated in a solvent of petroleum ether, ethyl ether, acetic acid (80:90:1, V/V). Bands separated by thin layer chromatography (TLC) containing fatty acid methyl esters (R_f 5.5–6.5) or monohydroxy steroids (R_f 0.10–0.15) were recovered and the lipids were eluted. The fatty acid procedures are described in detail in Bobbie and White (1980).

The steroids were derivatized by forming the trimethylsilyl ethers with N-trimethylsilylimidazole in pyridine (TSIM, Pierce Chemical Co. Rockford, Ill.) at 100 °C for 1 hr then exchanging with a mixture of heptafluorobutyl imidazole and heptafluorobutyric acid anhydride at 55 °C for 2 hr (Ikekawa *et al.* 1972).

The aqueous portion of the methanolysate was recovered and a portion was digested for analysis of lipid phosphate and phosphonate (White *et al.* 1979c). The internal standard of 1,2,4 butanetriol was added and the sample held at 60 °C in 1 N HCl for 2 hr in a screw cap tube. The acid was removed and the sugars were reduced in the presence of .13 N sodium borohydride in 0.05 N NaOH for 1 hr at room temperature. Methanol was added and the mixture neutralized and desalted on a column containing 2 g of Dowex 50 100/120 mesh in the acid form in a Pasteur pipette plugged with glass wool. The neutral alditols were recovered in 3 ml of water. The excess borate was removed in a stream of nitrogen after heating in methanol, the sugars were peracetylated in pyridine, acetic anhydride 1:1 V/V at 55 °C for 60 min. and analyzed by gas liquid chromatography (GLC). The amines and amino acids were eluted from the Dowex 50 column with 3 ml of 3 N HCl, the HCl was removed in a stream of nitrogen, the acids esterified in anhydrous methanol, concentrated HCl, chloroform (9:1:1, V/V) at 55 °C for 2 hrs, the liquids were removed in a stream of nitrogen, the amines and alcohols acetylated in pyridine: n-butyric anhydride (1:1, V/V) at 55 °C for 2 hr. The amines were then analyzed by GLC.

The residue of the lipid extraction was recovered, subjected to acid hydrolysis, purified by TLC and analyzed by GLC after formation of peracetylated nitrile derivatives as described by Fazio *et al.* (1979), except that butane-1-ol-4-amine was used as the internal standard in place of inositol.

Gas liquid chromatography (GLC)

Fatty acid methyl esters were analyzed on the Varian 3700 gas chromatograph with a model 8000 autosampler and CDS-111 data system. The esters were automatically injected using the splitless technique in hexane with 0.5 min venting time onto a Silar 10C (Applied Science, State College, Pa.) coated glass capillary, 0.24 mm I.D. and 50 m long that was programmed to increase temperature from 42 °C to 162 °C at 2 °C/min followed by a 30 minute isothermal period and a programmed 1 °C/min rise to 192 °C. The carrier gas was helium at an inlet pressure of 16 psi for a flow rate of about 1 ml/min. The detection was by flame ionization. The identification of the components was based on the chromatographic mobility on polar and non-polar glass capillary columns before and after hydrogenation and by electron impact-mass spectrometry (Bobbie and White 1980). The reproducibility, sensitivity and efficiency of separations have been described in detail (Bobbie and White 1980).

In the separation of the neutral carbohydrates the Varian 2100 gas chromatograph with flame ionization detection was utilized. The components were separated on a packed column of 100/120 mesh Supelcoport coated with 3 % SP-2330 (Supelco, Bellefonte, Pa.), 2 mm I.D. and 4 meters long, programmed at 2 °C/min from an initial 100 °C to 200 °C with nitrogen carrier gas flow of 32 ml/min. The sensitivity was 2×10^{-11} amps/mv. The injection volume of 2 μ l contained 19.2 mmoles of peracetylated 1,2,4 butanetriol (Aldrich Chemical Co., Milwaukee, Wis.) as internal standard. Components were identified by comparison of the retention time of authentic standards.

Amines and amino acids were separated on packed columns of 3 % SE-30 on 80/100 mesh on Gas-Chrom Q (Applied Science Laboratories, State College, Pa) 2 mm I.D., 2 m long, using a temperature program of 2 °C/min from an initial temperature of 100 °C to 200 °C with a nitrogen carrier gas flow rate of 25.5 ml/min.

Heptafluorobutyrate esters of the steroids were separated on 3 % SE-30 on Gas-Chrom Q with a nitrogen carrier gas flow rate of 13 ml/min run isothermally at 220 °C. Detection was by flame ionization. n-Triacontanol was used as an internal standard.

Adenine nucleotides

Added adenine nucleotides can be recovered quantitatively from the aqueous phase of a lipid extraction. In these experiments the alkaline-chloroform method of Tobin *et al.* (1978) was used. The lipid extraction is as efficient as the standard perchloric or sulfuric acid extractions in the recovery of adenine nucleotides from suspensions of *Escherichia coli* (Davis and White, unpublished experiment). The aqueous extract was then lyophilized, and the fluorescent 1-⁶N ethenoadenosine

derivative formed with chloroacetaldehyde and the $1\text{-}^6\text{N}$ ethenoadenosine nucleotides were separated with a phosphate buffer gradient by high pressure liquid chromatography and assayed by their fluorescence (Bobbie *et al.* 1979).

Fatty acid methyl ester nomenclature

Fatty acid methyl esters are designated as the number of carbon atoms in the chain: the number of double bonds, the position of the double bond nearest the omega (ω) end of the ester. Special designations are a prefix of a, i, br, Δ for anteiso, iso, branched or cyclopropane structures. Esters containing more than 18 carbon atoms in the alkyl chain with more than two double bonds are considered polyenoic fatty acid esters.

Monocultures

Wild type *Neurospora crassa* (a gift of Dr. A. G. DeBusk of Florida State University) was grown in Vogel's N broth (Vogel, 1956) at 35°C for 48 hr and then at 25°C for 6 days. The mycelial mat was harvested by filtration, quick frozen and lyophilized. *Escherichia coli* type B (the gift of Dr. J. H. Stuy of Florida State University) was grown in nutrient broth to stationary phase at 37°C , harvested by centrifugation, quick frozen, and lyophilized. Lyophilized estuarine fungi *Alternaria* sp. and *Fusarium* sp. were the gifts of Randle E. Hicks of the Institute of Ecology, University of Georgia, Athens, Georgia.

Estuarine detrital microbial populations

Strings of 100 5 cm square 2.5 mil polyethylene were incubated in Apalachicola Bay, North Florida, in the Spring of 1978 (White *et al.*, 1979d) for two weeks and then transferred with aeration to the laboratory in aerated tanks. In the laboratory the strings of plastic were incubated in 15 l of artificial estuarine water (Instant Ocean, 3‰ salinity for 7 days at 25°C with aeration at ambient laboratory light levels. A control was maintained without supplementation. Eukaryotic growth was stimulated in another tank by daily supplementation of streptomycin 50 mg/l and penicillin 4 mg/l with 0.1 mM sucrose and 0.05% W/V nutrient broth (Difco Products, Detroit, Mich). The pH was maintained at 5.5 with HCl. In the third tank prokaryotic growth was stimulated by adding daily 0.25 mg/l cyclohexylimide with 0.01 mM NaH_2PO_4 and 0.1 mM glutamine at a pH maintained at 7.8. After 7 days the strings were removed and analyzed.

Radioactive uptake

Plastic leaves taken from the manipulations of the estuarine detrital microbial experiments after 7 days exposure were incubated with 1 mCi $\text{H}_2\text{}^{32}\text{PO}_4$ and 100 μCi $\text{H}_2\text{}^{35}\text{SO}_4$ and samples were recovered at 2 hr intervals for 10 hr, the lipids were extracted and dissolved in toluene and the radioactivity determined (King *et al.*

1977). Triplicate samples were counted, the data were plotted on semilog paper and the times necessary to double the incorporated isotope recorded. Incorporation of ^3H -methyl thymidine into DNA was measured on plastic leaf samples by exposure to 2 $\mu\text{Ci}^3\text{H}$ methyl thymidine, alkaline extraction, purification, and hydrolysis of the DNA (Tobin and Anthony 1978) and is expressed as the time necessary to double the incorporation.

Respiration

The rate of oxygen utilization by the microbes on the plastic surfaces was measured polarographically at 25°C (Morrison *et al.* 1977).

Results and Discussion

Fungi - bacteria lipid differences

A series of reciprocal mixtures of lyophilized *E. coli* and *N. crassa* were extracted and subjected to the analytical sequence diagrammed in Figure 1. Characteristic gram-negative bacterial fatty acids such as $\Delta 17:0$ follow the same trend as the cis-vaccenic acid ($18:1\omega 7$) illustrated in Figure 2. Polyenoic fatty acids found only in eukaryotes increase linearly as the proportion of *Neurospora* is increased (Fig. 2). The amounts of long chain

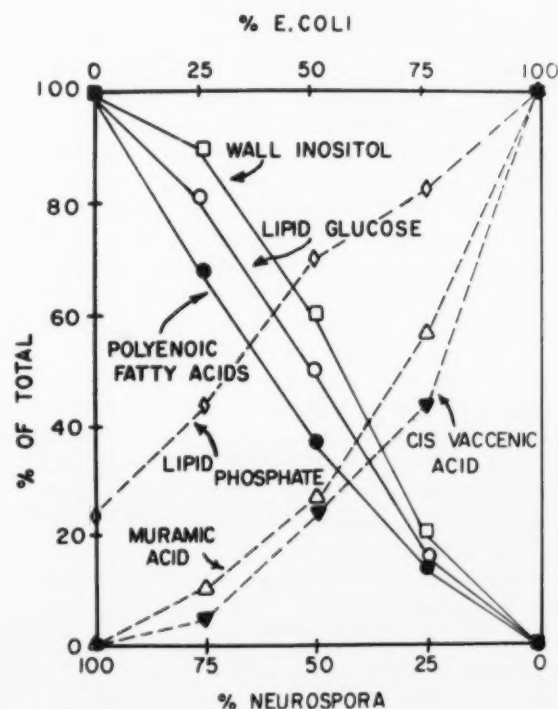


Fig. 2. Analysis of lipids from reciprocal mixtures of *Neurospora crassa* and *Escherichia coli*. Data are given as % of the total component recovered in the pure bacteria or fungus. (Δ - Δ) muramic acid; ($\circ$ - $\circ$) lipid glucose; ($\square$ - $\square$) wall inositol; (∇ - ∇) cisvaccenic acid; ($\bullet$ - $\bullet$) polyenoic fatty acids; ($\diamond$ - $\diamond$) total lipid phosphate.

fatty acids like 24:0 match the changes in proportions of the polyenoic fatty acids. With increasing proportions of *Neurospora* the lipid glucose values increase (Fig. 2) and match changes in lipid inositol, galactose, mannose and xylose. *E. coli* does not contain lipid neutral carbohydrates. Many other bacteria contain lipid carbohydrates (Shaw 1974).

Analyses of amines and amino acids show increasing content of lipid serine as the proportion of *Neurospora* increases. *E. coli* contains a trace of phosphatidyl serine, but eukaryotes generally contain a higher proportion of this lipid.

The total lipid phosphate increases linearly as the proportion of *E. coli* in the mixture increases, reflecting the high phospholipid content of Eubacteria (Fig. 2). The total lipid glycerol increases as the proportion of *Neurospora* increases, again reflecting the higher content of neutral lipids.

The residue remaining after lipid extraction contains the aminoglucoside muramic acid, which is a unique component of the prokaryote cell wall. It increases as the proportion of *E. coli* increases (Fig. 2). Complex inositols are found in fungal cell walls and increase as the proportion of *Neurospora* increases (Fig. 2). Glucosamine is found in the walls of both bacteria and fungi, but the proportion is higher in fungi and the residue glucosamine increases as the proportion of *Neurospora* increases. The 200 mg (dry wt) *E. coli* sample contained $.054 (\pm 0.014)$ μ moles of glucosamine, compared with $1.04 (\pm 0.12)$ μ moles in 200 mg of *Neurospora*.

These experiments indicate the possibility that analysis of the lipids and lipid extrate residue can be used to define the community structure of microbial assemblies. To test this possibility a detrital microbial community that colonized plastic surfaces in a subtropical estuary was brought to the laboratory and incubated under conditions that favor prokaryotic growth and inhibit eukaryotic growth (glutamine, phosphate, pH 7.8 and the eukaryotic inhibitor, cyclohexylimide) or conditions favoring eukaryotic fungal growth and inhibition of prokaryotic growth (nutrients sucrose, pH 5.5 and the antibiotics penicillin and streptomycin). After 7 days the plastic surfaces were harvested and the microbial morphology examined by scanning electron microscopy.

The detrital microbial community in which the growth of eukaryotes was stimulated shows the clear presence of mycelia (Fig. 3, upper panel), which are absent from the community where prokaryotic growth was stimulated in the presence of cyclohexylimide (Fig. 3, lower panel). The control shows the morphology of both eukaryotes, fungal mycelia and algae as well as typical bacteria. Addition of antibiotics, acid, and complex nutrients enhanced fungal overgrowth. Cyclohexylimide, alkaline pH, and low nutrient concentrations enhanced bacterial growth with typical prokaryote morphology.

The extractable lipid phosphate has been shown to be a good measure of microbial biomass (both prokaryotes and microeukaryotes) in sediments and detritus (White *et al.* 1979a). The extractable lipid phosphate is increased 10-fold in the eukaryote-stimulated and 2-fold in the prokaryote-stimulated microcosms when compared to the control (Tab. I A). The increased lipid phosphate in the supplemented community is reflected in similar increases in the respiratory activity measured by oxygen utilization and the extractable adenosine nucleotides. These measurements have been shown to have significant correlations with the estuarine detrital microbial biomass (White *et al.* 1979a).

Simple hydrolytic manipulations of the phospholipids can give information about the microbial population structure (White *et al.* 1979b). Phosphonolipids with the C-P bond are formed uniquely by some microeukaryotes (Kittredge and Roberts 1969, Lechevalier 1977). In this experiment only by stimulating fungal growth and inhibiting bacterial growth were they detectable as a significant portion of the lipid phosphate (Tab. I A). The proportion of the lipid phosphate in diacyl phospholipids is very high in simple Eubacteriaceae and approaches 50% of the lipid phosphate in structurally more complex prokaryotes and eukaryotes (White *et al.* 1979b). The proportions of phospholipids in all three microcosms was nearly equal and was typical of a mixed community of microeukaryotes, complex prokaryotes and the Eubacteriaceae (Tab. IA).

Muramic acid is a unique component of the prokaryote cell wall (King and White 1977, 1978). Statistically insignificant differences (t-test) were observed between control and prokaryote-stimulated microcosms. Both control and prokaryote-stimulated microcosms show twice the level of muramic acid in the eukaryote-enriched microcosm (Tab. I A).

Rates of ^{32}P incorporation into phospholipid and ^3H -thymidine incorporation into DNA have been shown to correlate with other measures of growth rate in estuarine detrital microflora (White *et al.* 1977, 1979c). The incorporation rates of ^{32}P into phospholipid and ^3H -methyl thymidine into DNA in the prokaryote-stimulated microcosm are roughly twice the rates in the eukaryote-stimulated system (Tab. I A). These rates are related to the true average rate for the populations but the true values may be very different, since they depend critically on the specific activities. The increased rate of phospholipid and DNA synthesis in the prokaryote-stimulated environment is all the more impressive as the biomass (measured as lipid phosphate) is less than half that in the eukaryote-stimulated environment.

The incorporation of ^{35}S into lipid sulfate is nearly equal to the rates of ^{32}P into phospholipids in the

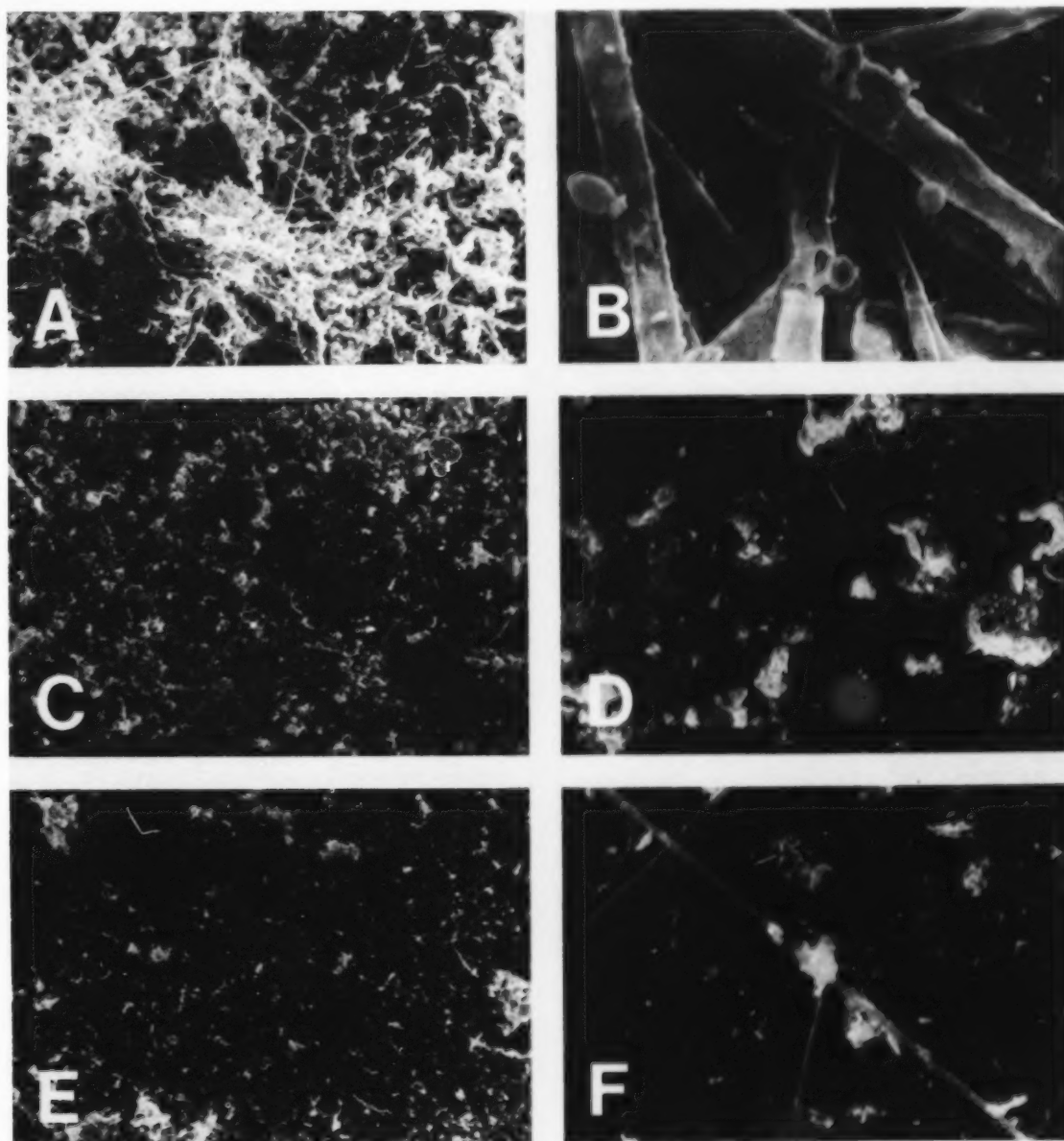


Fig. 3. Scanning electron micrographs of the estuarine detrital microbial community after 7 days of incubation in aerated estuarine water to which the following components (expressed in final concentrations) were added. Upper panel A,B: streptomycin 51 mg/liter, penicillin 4 mg/liter; 10 μ moles sucrose, and 0.01 % nutrient broth (W/V) maintained at pH 5.5; middle panel C,D: control without supplementation, lower panel E,F: cyclohexylamide 25 mg/l, 10 μ M NaH_2PO_4 , 100 μ M glutamic acid maintained at pH 7.8. Figs. 3A, 3C, 3E are at 200X; Figs. 3B, 3D, 3F are at 2000X.

control or prokaryote-stimulated environments but is more rapid than the incorporation of ^{32}P into the phospholipids of the eukaryote-stimulated environment (Tab. IA).

Ratios of components identified with specific portions of the microbial community compared with the total microbial biomass have shown excellent correlation with morphology by scanning electron microscopy

during succession in the estuarine detrital microflora (Morrison *et al.* 1977). The initial bacterial colonizers showed a low ATP/muramic acid ratio that was progressively replaced by a morphologically more complex population with an increasing ATP/muramic acid ratio. Lipid phosphate is possibly a more accurate measure of biomass than extractable ATP unless both ADP and AMP are measured. This is because the ATP is metabolically labile and rapidly hydrolyzes to ADP and AMP

Tab. 1. Comparison of components and activities of the estuarine detrital microflora incubated under conditions to stimulate or repress microeukaryotes or prokaryotes. Eukaryote stimulating conditions produced the community shown in Fig. 3, upper panel; prokaryote-stimulating conditions produced the community shown in Fig. 3, lower panel. Where indicated, values are given as $\bar{X} \pm (SD)$ for 5 determinations; lipid classes are given as % of total lipid phosphate; times for 2-fold increase in isotope incorporation are related to the growth rate but are relative as they depend on the specific activity; adenosine nucleotides = AMP+ADP+ATP.

A				D			
Microbial biomass and activity				Neutral carbohydrates			
Measure	Eukaryote-stimulated	Control	Prokaryote-stimulated	Ratio of components to 1,2,4 butanetriol	Eukaryote-stimulated	Control	Prokaryote-stimulated
Oxygen uptake $\mu\text{moles O}_2/\text{hr}/\text{leaf}$	0.76	0.065	0.11	A	0.04	—	0.06
Lipid phosphate $\mu\text{moles}/\text{leaf}$	0.29 (.04)	0.05 (.008)	0.07 (.003)	Glycerol	0.96	0.12	1.02
% phosphonolipid	50.4 (4)	0	0	B	0.07	0.07	0.26
% diacylphospholipid	68 (2)	65 (2)	62 (1.5)	C	0.91	0.26	2.33
Muramic acid nmoles/leaf	1.92 (.3)	3.4 (1.0)	2.96 (.6)	D	0.12	0.03	0.15
Lipid ^{35}S incorporation (time for 2-fold increase in ^{35}S)	6.2	2.8	5.0	E	0.22	0.03	0.56
Lipid ^{32}P incorporation (time for 2-fold increase in ^{32}P)	9.1	2.3	4.25	F	0.05	0.01	0.05
^3H incorporation into DNA (time for 2-fold increase in DNA)	1.5	—	0.6	G	0.02	—	0.09
Adenosine nucleotides nmoles/leaf	1.2 (.05)	0.7 (.03)	0.9 (.03)	H	0.01	0.01	1.10
				I	0.09	—	0.07
				J	0.04	—	0.01
				Rhamnose	0.01	0.01	0.04
				Fucose	0.06	0.003	0.01
				K	0.07	—	0.30
				Ribose	0.03	0.03	0.22
				Arabinose	0.02	0.01	0.42
				L	—	0.03	—
				M	0.14	0.19	0.18
				Xylose	0.03	0.001	0.39
				N	0.07	0.03	0.35
				Mannose	0.01	—	—
				Galactose	0.01	0.01	0.04
				Glucose	0.04	0.01	0.19
				Inositol	0.40	0.01	0.03
B				E			
Molar ratios of components				Lipid amines and amino acids			
Ratios	Eukaryote-stimulated	Control	Prokaryote-stimulated	Ratio	Eukaryote-stimulated	Control	Prokaryote-stimulated
Lipid phosphate/muramic acid	0.16	0.015	0.024	Ethanolamine	—	0.02	0.14
Adenosine nucleotides/muramic acid	0.63	0.21	0.30	Serine	0.08	0.04	—
Adenosine nucleotides/lipid phosphate	0.004	0.014	0.013	Lysine	0.24	0.28	0.14
Lipid glycerol/lipid phosphate	1.6	1.2	7.1				
Lipid ^{35}S /lipid ^{32}P incorporation	0.7	1.2	1.2				
Ergosterol/lipid phosphate	0.0002	0.00005	—				
C				F			
Ratios of fatty acid methyl esters				Steroids			
Ratio	Eukaryote-stimulated	Control	Prokaryote-stimulated	Ratio of steroids to triacontanol	Eukaryote-stimulated	Control	Prokaryote-stimulated
a + i 15:0/15:0	0.85	0.78	2.3	A	0.013	0.012	0.1
18:1 ω 7/18:1 ω 9	0.44	1.15	3.0	Cholesterol	0.067	0.001	—
16:1/16:0	0.35	0.41	0.59	Ergosterol	0.350	0.013	—
18:2 ω 6/18:0	1.53	0.47	0.24	B	0.340	—	—
Δ 17:0/19:0	0.001	0.07	0.11	C	0.003	—	—
Δ 19:0/19:0	0.007	—	0.04	D	0.04	—	—
20:5 ω 3/19:0	0.22	0.04	0.05	E	0.05	—	—
22:6 ω 6/19:0	0.02	—	0.007	F	0.025	0.008	0.011
Polyenoic/total	0.12	0.08	0.02	Triaccontanol	1.00	1.00	1.00
24:0	0.10	0.05	0.05	G	0.004	0.002	0.076
				H	0.003	0.018	0.020
				I	—	0.005	0.021

(Goldenbaum *et al.* 1975). The technique reported here measures all three nucleotides. The lipid phosphate to muramic acid ratio is lowest in the control and prokaryote-stimulated microcosm, suggesting that a greater proportion of the biomass contains muramic acid. As expected, the eukaryote-stimulated microcosm has a higher ratio of lipid phosphate to muramic acid (Tab. I B). The same is true for the ATP to muramic acid ratios (Tab. I B). The ratio of adenine nucleotides to lipid phosphate is highest in the prokaryote-stimulated microcosm indicating that a lesser proportion of the biomass contains a high phospholipid content (Tab. I B). The glycerol to phosphate ratio approaches 1 in simple gram-negative eubacteria (as in *E. coli*) where the major lipid is phosphatidyl ethanolamine. However, in many other bacteria the major lipids

are derivatized phosphatidyl glycerol or glucosyl glycerides. In eukaryotes there can be high concentrations of glycerol-containing neutral lipids. In these experiments inhibition of eukaryotic growth increased the lipid glycerol to phosphate ratio over the prokaryote-inhibited or control microcosms (Tab. IB). The lipid classes could be differentiated (King *et al.* 1977) to increase the diagnostic utility of this ratio in determining the microbial community structure.

The ratio of the rate of sulfolipid synthesis to the growth rate (phospholipid synthesis or DNA synthesis) is greater in the eukaryote-enriched population (Tab. IB).

The diverse fatty acid methyl esters derived from the lipids of the detrital microflora can be separated by the extraordinary resolving power of glass capillary (GLC). Figure 4 illustrates the gas chromatogram of the fatty

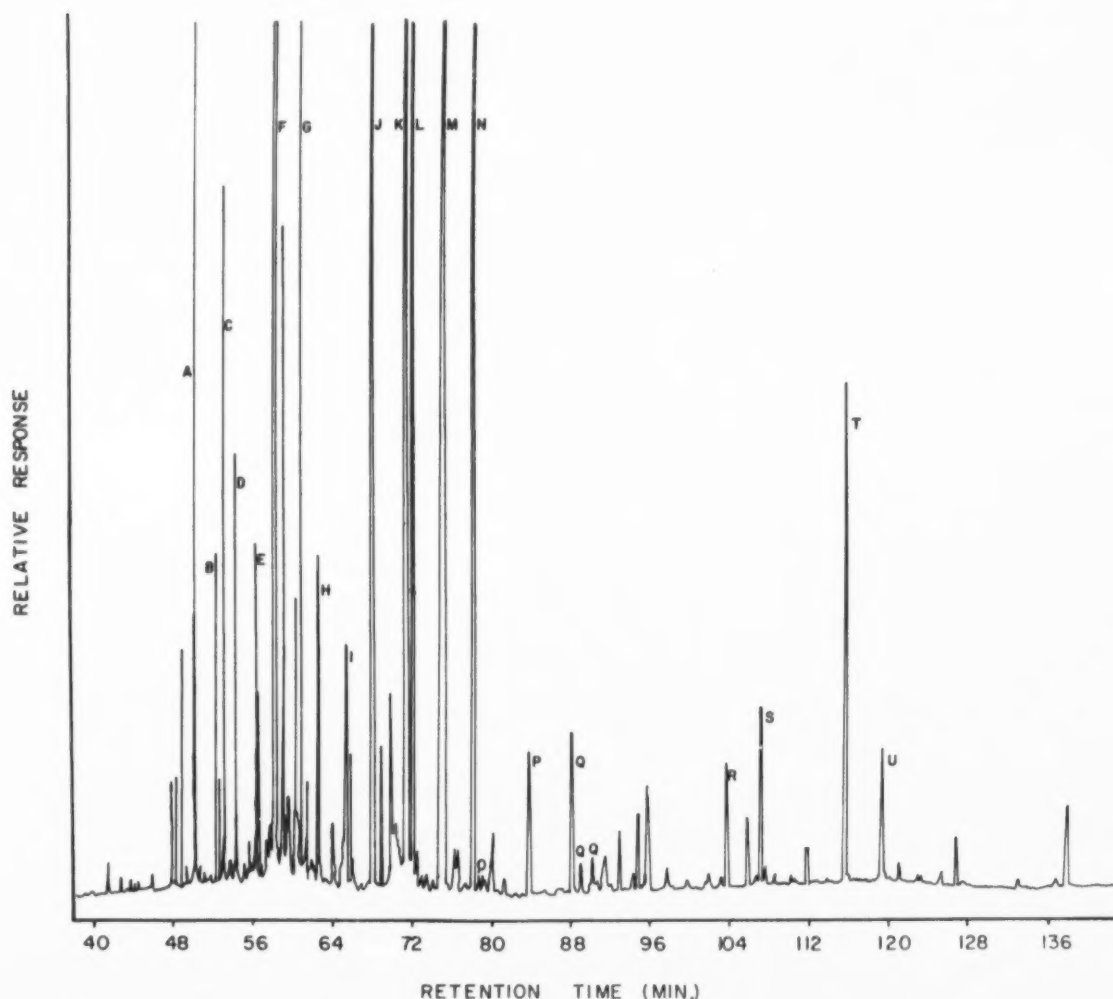


Fig. 4. Gas chromatogram of fatty acid methyl esters from the lipids of the control detrital microbial population separated on a 50 m silar 10C glass capillary column. A = 14:0; B = i 15:0; C = a 15:0; D = 15:0; E = i 16:0; F = 16:0; G = 16:1; H = 17:0; I = 17:1; J = 18:0; K = 18:1 ω 9; L = 18:1 ω 7; M = IS 19:0; N = 18:2 ω 6; O = Δ 19:0; P = 20:0; Q = 20:1 br; R = 22:0; S = 22:1; T = 20:5 ω 3; U = 24:0. IS = Internal Standard.

acid methyl esters derived from the control community. The usefulness of fatty acid methyl esters in helping to define the community structure of the detrital or sedimentary microbes has been discussed (Bobbie and White 1980, White *et al.* 1979b). From the data illustrated in Table I C a large increase in the ratio of the branched 15:0, the cyclopropane $\Delta 17:0$, $\Delta 19:0$ fatty acids and the cisvaccenic acid 18:1 $\omega 7$ formed by the prokaryote anaerobic pathway are detected in the prokaryote-stimulated population.

The eukaryote population shows large increases in the 18 carbon dienolic, the $\omega 3\alpha$ linolenic and $\omega 6\gamma$ linolenic polyenoics and the 24:0 long chain fatty acid esters. Ratios of α and γ linolenic polyunsaturates are different in different classes of fungi (Erwin 1973). Higher fungi contain the α linolenic series. Lower fungi contain both, thus the high resolution possible with this system could give further differentiation to the population structure.

The aqueous portion of the acid methanolysate contains components that can help differentiate the community structure. Unknown components H, K, N and ribose, arabinose and glucose are significantly increased in the prokaryote-stimulated community (Tab. ID). Lipid inositol is greatly enriched in the fungal enriched community (Tab. ID). The resolution and retention volume of known and unknown neutral carbohydrates derived from the lipids and separated by GLC are illustrated in Figure 5.

The lipid amino acids and amines show high levels of ethanolamine (presumably from phosphatidyl ethanol-

amine and lysine (presumably from lysyl phosphatidyl glycerol) in the prokaryote-enriched microcosm and the control (Tab. I E). Serine, presumably from phosphatidyl serine, is enriched in the eukaryote-stimulated microcosm and is present in the control. Lipid lysine is also found in the eukaryote-stimulated microcosm.

Steroids

The steroids recovered from the TLC plate were analyzed by GLC after formation of the heptafluorobutyl esters (Tab. IF). The eukaryote-stimulated microcosm contained 10 to 50 times more of the cholesterol, ergosterol and a steroid with a retention volume just less than the sitosterol ester than the control. In the prokaryote-stimulated environment few steroids were detectable. The steroid levels are low, representing 0.02% of the eukaryote stimulated microbial assemblies' phospholipid when calculated on a molar basis. The control contained a ratio one-fourth of the eukaryote-stimulated microbes (Tab. IB).

Estuarine fungi

Two estuarine fungi were isolated from *Spartina alterniflora* detritus and cultured and identified by Randle E. Hicks of the University of Georgia. The total phospholipid and fatty acid composition of the two estuarine fungi were similar (Tab. II A) and the fatty acid composition like the *Neurospora* used in the data of Figure 2. *Neurospora*, however, contained four times the phospholipid per gram dry weight. The lipid carbohydrates from both estuarine fungi contained large amounts of component N (Fig. 5) and xylose, but differed in the content of inositol, mannose and in the lipid glycerol to phosphate ratio. Analyses of this type on fungi grown and isolated under different conditions will greatly help in the interpretation of data on the lipid composition.

From the data in Figures 2 and 3 and Table I, it appears that biochemical measures which have much less selective bias than cultural methods may be effective predictors of the detrital microbial population structure. The analysis of mixtures of *E. coli*, the everyman bacteria, and *Neurospora* as a representative fungus, gave lipid and wall components that allow clear differentiation. From the data of Figure 2, the wall inositol, rather than glucosamine, would be a better measure of fungal biomass. Judging from the comparative data given by Weete (1974) possibly the best measure of fungal biomass will be the ergosterol-like steroids. With the hydrolysis and assay of the isolated components by the powerful resolving capabilities of GLC, nonspecific colorimetric analyses can now be superseded by more specific and sensitive analyses. Manipulation of the estuarine detrital microflora by changing the water column chemistry did produce populations enriched in fungi which showed ratios of biomass (lipid phosphate and adenosine nucleo-

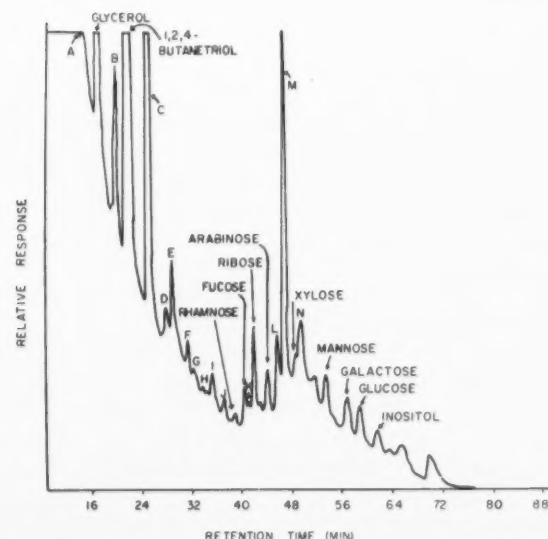


Fig. 5. Gas chromatogram of the lipid neutral carbohydrates alditol acetates from the control detrital microbial population separated on a packed column SP-2330. 1,2,4 butanetrioltriacetate is the internal standard.

tides) to muramic acid that were higher, slower relative rates of DNA and phospholipid synthesis, the presence of phosphonolipids, lower proportions of phospholipids, higher relative rates of sulfolipid synthesis (Tab. I A, B) and a morphology typical of fungi (Fig. 3). Lipid analysis showed the higher long chain 18:2, and polyenoic fatty acids, higher lipid inositol, serine and sterols (Tab. I), than the prokaryote-enriched population. The relatively rich lipid neutral carbohydrate spectrum in the prokaryote-enriched assembly was not found in the initial *E. coli* - *Neurospora* experiment (Fig. 2), but is typical of many other bacteria (Shaw 1974). Short iso and anteiso branched fatty acids not found in *E. coli* are also considered typical of many other bacteria (Schultz and Quinn 1972, 1973, Bobbie and White 1980), and are enriched in the prokaryote-stimulated microcosm.

Steroids with the rich diversity known in the fungi (Weete 1973, 1974, Wassef 1977, Rattray *et al.* 1975) should allow differentiation between major groups of fungi. Steroids are found in relatively low concentrations even where eukaryotic fungal growth was stimulated (Tab. I B). The extraordinary resolving capacity of glass capillary gas chromatography when combined with the quantitative formation of the heptafluorobutyl esters which forms a more volatile and intense responding derivative that activates the extremely sensitive electron capture detector may make steroid analysis like that illustrated in Table IF a good measure of fungal community structure. The structural identity of the components separated by GLC, can often be resolved by combined gas chromatography, mass spectrometry. In these environmental studies this technique has thus far only been applied to the major fatty acid methyl esters (Bobbie and White, 1980).

The methods outlined in this study can be used to delineate fairly clearly the prokaryotic and microeukaryote portions of the community. The application of these techniques to differentiate the contribution of different classes of microeukaryotes, the algae, the protozoa, the fungi or the microinvertebrates, may require a more comprehensive analysis where intact lipids are separated before methanolysis and analysis. The newly developing technique of high pressure liquid chromatography with fluorometric derivatization may prove extremely useful in these initial separations. As the analysis of more and more components of the microflora are completed (as indicated in Tab. II) unique components may allow the contribution of individual species to be analyzed.

Studies of microbial colonization of fresh vascular plant detritus using these methods would be complicated by the presence of lipid components from the detritus. To differentiate the lipids from the microbial community, the detritus and its microflora would need to be incubated under conditions where isotope incorporation into the microbial lipids would be stimulated

Tab. II. Comparison of the lipid components of *Fusarium* sp. and *Alternaria* sp.

Component	<i>Fusarium</i>	<i>Alternaria</i>
Lipid phosphate μmoles/200 mg	0.57	0.57
A		
Fatty acid composition		
14:0	0.031	0.01
15:0	0.007	0.006
16:0	1.00	1.00
16:1	0.06	0.03
17:0	—	0.016
18:0	0.32	0.39
18:1ω9	2.02	2.01
18:1ω7	—	0.07
18:2ω6	0.88	1.05
18:3ω3	0.009	0.005
20:0	0.03	0.013
20:0	—	0.024
B		
Ratios of components to 1,2,4 butanetriol		
Neutral carbohydrates		
Glycerol	2.5	0.06
B	0.001	—
Arabinose	—	0.002
L	0.04	0.16
Xylose	0.11	0.14
N	0.11	0.16
Mannose	0.008	—
Inositol	—	0.009

and the analyses would require an efficient isotope detector such as a mass spectrometer operating in the selective ion mode.

The ecological importance of this type of detailed analysis of the detrital microbial population structure has been demonstrated in experiments where the mass, species and number of microbial predators attracted to various types of detritus incubated in an estuary were measured over a period of time (White *et al.* 1979d). There was no significant correlation between measures of microbial biomass such as oxygen utilization, lipid phosphate, or the amount of the endogenous prokaryote storage product poly-β-hydroxybutyrate with the number of species, number of individuals, biomass, or indices of the macrobial detritivores attracted to the detritus. However the analysis of the microbial fatty acids showed surprisingly good statistical significance between the content of branched 15:0 and Δ17:0 fatty acids in the microflora and the biomass, number of species and Margalef richness of the detritivores attracted to the detritus. This suggests the animal predators sense the bacterial components of the microflora to a greater degree than the total microbial biomass.

Acknowledgments

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Discussion

- Lopez:** What biochemical methods do you think would be useful in distinguishing living microorganisms from microbial debris that might be building up in the environment?
- White:** The most definitive method for distinguishing the living sedimentary microflora involves labeling an isolated portion in a vessel then incubating in the presence of an unlabeled precursor in a pulse-chase type of experiment such as that illustrated in our work with the lipid metabolism of detritus (King, White and Taylor. 1977. *Applied Environmental Microbiology* 33: 1177–1183). The changes in radioactivity of particular components tell a great deal about the composition and activity of the sedimentary microbes. Short of isolating the sediment, a measure of the physiological status of the microbes can be gained by noting the "energy charge" $ATP + \frac{1}{2} ADP/ATP + ADP + AMP$ which gives excellent correlation with the growth status (Knowles, C. J. 1977. *Symp. Soc. Gen. Microbiol.* 27: 241–283), or the ATP/GTP ratio which correlates with the rate of macromolecular biosynthesis (Karl, D. M. 1978. *Applied and Environmental Microbiology* 36: 349–355). The nutritional status of the microbes can often be correlated with the poly- β -hydroxybutyrate level (Herron *et al.* 1978. *Applied and Environmental Microbiology* 35: 251–257).
- Lopez:** Is there a way to find out which components of the detritus the microbes are using and which microbes are using it?
- White:** The way to follow the catabolism of detritus is to use labeled detritus in the way Ken Tenore is doing at Skidaway (1977. *Marine Biology* 44: 51–55). There are ways to determine which of the microbial population in the water column are active either by radioautography, epifluorescence-direct counting with radioautography (Meyer-Reil, L. A. 1977. *Microbial Ecology of a Brackish Water Environment*, Springer-Verlag, p. 223–230), or by distinctive changes in shape produced by incubation with nadilixic acid and observed by epifluorescent counting. (Canad. J. Microbiol. 25: 415–470, 1979) These procedures seem to work best with the water column microbes.
- Sansone:** Have you compared your liquid chromatography method for measuring adenosine phosphates with the luciferase method?
- White:** Yes
- Sansone:** And they are comparable?
- White:** Yes.